SOME PROPERTIES OF AN ACID TOLERANT AZOTOBACTER, AZOTOBACTER INDICUM

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By

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The author (1) has previously isolated an acid-tolerant azotobacter from an acidic volcanic ash soil in Tohoku district in Japan, applying Winodradsky's soil plate technique. From its diagnostic characters the organism was identified as *Azotobacter indicum* Starkey et De. As is well known, *A. indicum* is distinctive in some respects from other *Azotobacteriaceae* species such as *A. agile* and *A. chroococcum*, and indeed its small size, slow growth, vigorous slime production, and especially its enhancing capacity in acidic media are characteristically mentioned. The organism, on the other hand, has been once generally assumed to restrict its favorite habitat only over tropical regions like India as well as Java, and accordingly its properties are invenstigated not so thoroughly as are those of other azotobacters. The author examined its growth and slime production in various media, especially in the media containing extra nitrogen matters. The results obtained are presented here.

Materials and Techniques

Organism. A strain of *A. indicum*, previously isolated from an acidic volcanic ash soil by the soil plate technique and purified by repeated N-free agar platings, was used as a test organism throughout the work.

Media. 100 ml or 7.5 ml of Starkey's salts solution (the basal medium) supplemented with a carbon compound tested was distributed in a 500 ml Sakaguchi's flask or a somewhat modified Ogata's shake tube (2), illustrated in Fig. 1, respectively, and autoclaved at 15 lb for 10 min. When if necessary, nitrogenous substances were added before autoclaving.

Culture. 0.5–1 per cent of fully grown liquid culture was transferred to a new medium with a sterile pipette. Flasks or tubes so inoculated were set on a reciprocating shaker in a chamber of 30°C.

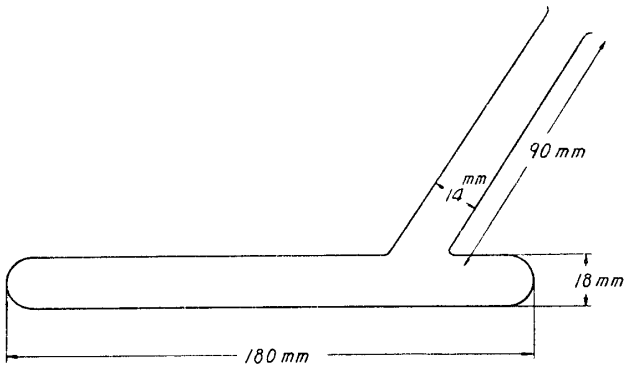

Fig. 1. Modified Ogata's shake tube

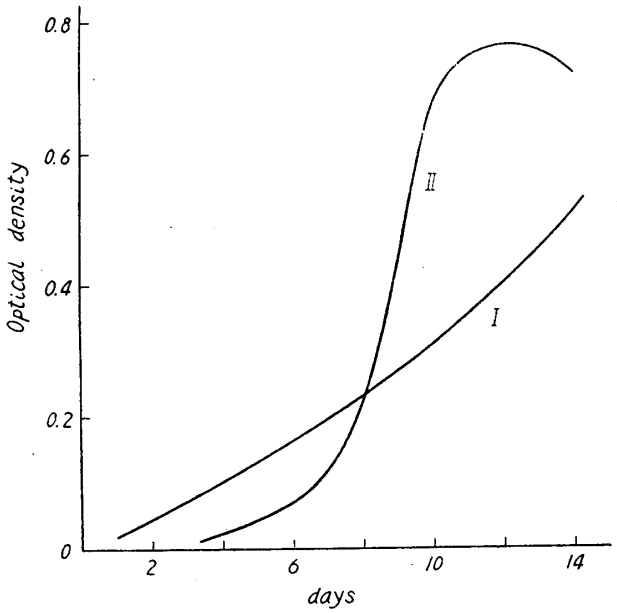

Fig. 2. Growth curves in N-free medium
I. stand culture,
II. shake culture

Supplementary descriptions of the technique, if necessary, were presented in the part of Results.

Experimental Results

A) Utilization of Carbon Compounds

The growth of the organism was expressed with optical density of the culture measured directly in Ogata's shake tube with an electricphotometer using a filter of 570 mμ. The growth of the organism in a medium containing 1 per cent of glucose proceeds as illustrated in Fig. 2. When cultured with shaking, it shows a distinct induction period of 4–5 days and after that period bursts into a rapid proliferation, and the growth ends within 48 hours or so. When, on the contrary, it is stand-cultured, it grows very slowly though without any appreciable induction period and requires almost 20 days to complete its growth. The similar phenomenon has been also observed by Sato (unpublished) when a halophilic yeast, a slow grower, isolated from "Shoyu", is cultured in Koji extract containing a heavy concentration of table salt. Then the shake-culture technique is convenient to detect growing aspects of the organism because of an establishment of the notable induction period following rapid growth, and accordingly adopted throughout the work.

Glucose, sucrose, mannose, fructose, galactose, maltose, lactose, xylose, arabinose, mannitol, butyrate, acetate, lactate, pyruvate, succinate, and benzoate, respectively, were tested to establish a suitable carbon source. Glucose, mannose, galactose, fructose, sucrose, mannitol, butyrate, lactate, and pyruvate are utilizable and all of them showed distinct induction periods of different

length. The results are summarized in Table 1. Jensen (3) stated that *A. chroococcum* and *A. agile* were not reported to utilize mannose and, however, he did not refer to *A. indicum*. And our strain utilizes mannose. It requires a longer induction period for the utilization of mannitol than glucose. Pentoses were not utilized by the organism like other azotobacter species, and it might be notable to mention that our species lacked in utilization capacisty of acetate, succinate, and benzoate in contract to other azotobacter species.

Table 1. Growth substrate and length of induction period

Exp. No.	Substrate	Induction Period (Days)	Exp. No.	Substrate	Induction Period
1	Glucose	5.0	2	Glucose	3.5
	Sucrose	4.5		Na-Butyrate	4.5
	Galactose	5.0	3	Glucose	4.5
	Fructose	6.5		Na-Pyruvate	4.5
	Mannose	9.0		Na-Lactate	5.0
	Mannitol	9.5			

When the organism was cultivated in the media containing either of the above utilizable substances, all medium then grown became very viscous at the end stage of the culture. It is well known that most of azotobacter species produce slime more or less which may play an important role in cementing soil particles in the field. Then, the slime produced abundantly by our strain was subjected to examine.

B) Properties of Slime

a) Separation of the slime. Maximal growth of the organism was attained at the end of a week when cultured with shaking, and the grown medium became viscous also to maximal extent. The viscous culture of such a rather fresh age offered a difficulty to separate the cells by centrifugation, and when dilution was allowed in order to make separation more easily, the resultant supernatant liquid so obtained almost lost its viscous nature, so that there occured a little precipitation by the addition of a solvent such as acetone and only scanty volume of the slime was obtainable. It seems then that the slime contained in such a culture adhered to the cell tightly. But when two weeks old culture was used for the material of the slime and centrifuged at 10,000 rpm for 40 min after addition of 3-4 volumes of water, almost clear but enough viscous supernatant was thus obtained. 4-5 volumes of acetone were added to the supernatant and the resultant slime matter floated as a floculate containing air-bubbles, and it was then seperated by centrifugation and re-disolved in water and reprecipitated with acetone. The precipitate was dried in a vacuum descicator. The crude slime preparation was light brown or light dirty green scales with an appearance of gelatinous flakes. They were not

hygroscopic but readily dissolved in water. The aqueous solution of the slime was almost translucent and very viscous, and its viscosity was measured with Ostwald's viscosimeter in the bath of 30°C.

b) Components of the slime. The results of the chemical analysis were indicated in Table 2. Ash content is very high while nitrogen content is very

Table 2. Chemical analysis of crude slime

Moisture	Total N	Carbohydrate (as glucose)	Ash
4.23 %	0.48 %	57 %	9.57 %

low. After the acid hydrolysis, the hydrolysate showed the reducing ability against triphenyltetrazolium-chloride (TTC) at the alkaline reaction. When the reducing substance was estimated as glucose, its content was only 70 per cent of the remaining portion other than moisture, ash and crude protein calculated from Kjeldahl nitrogen.

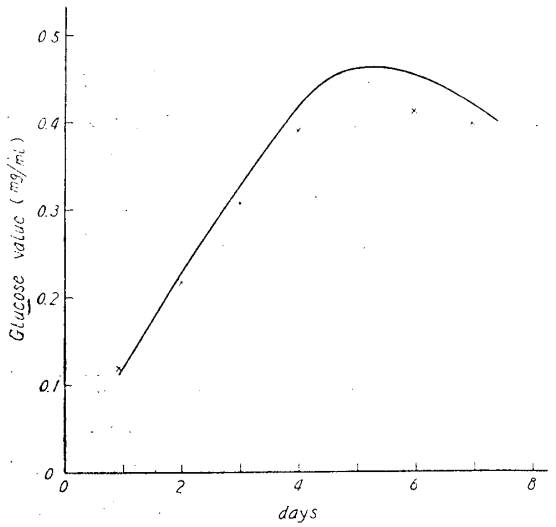

Fig. 3. Reducing ability of slime hydrolysate and time of heating.

When 2N H_2SO_4 solution of the slime in sealed tubes was heated in a boiling water bath, the reducing ability of hydrolysates varies with time. An example of the experiments is shown in Fig. 3. The viscosity of the solution fell down to the level of water after 3 minutes' heating, but reducing activity appeared only after an hour, which attained to the maximum after 5 hours and thereafter decreased gradually.

Then the slime was hydrolysed in 2N H_2SO_4 for 5 hours, and the hydrolysate was neutralized with $Ba(OH)_2$ and also sulfuric acid was eliminated. The filtrate and washings of the precipitated $BaSO_4$ were combined together and evaporated to dryness in vacuo. This hydrolysate was tested with the color reactions, as listed in Table 3. From the positive result of naphthoresorcinol reaction uronic acid may be present. From the negative results of Seliwanoff and Pinoff reaction the existence of keto sugar may be denied. From the positive results of orcinol or aniline reaction there may be recognizable pentose or uronic acid that shows the similar reactions with pentoses. Color due to phloroglucinol reaction is brown, and resembles that of glucose,

Table 3. Color reactions of slime hydrolysate.

Reagent	Reaction	Color	Color of slovent layer
Molisch	+		
Naphthoresorcinol	+	Redviolet →Dirty green	Alcohol Green / Ether Red violet / Benzol Light red violet
Seliwanoff	−		
Pinoff	−		
Orcinol	+	Blue	Amyl alcohol Blue
Aniline	+		
Phloroglucinol	+	Brown	Amyl alcohol Brown
Acetone	−		
Ninhydrin	−		

since arabinose indicates red-violet color and xylose dark blue one. A methylpentose may not be contained as the acetone reaction is negative. It is noteworthy to mention that ninhydrin reaction is negative, while slime preparation contains 0.48 per cent of Kjeldahl nitrogen. If such nitrogenous substances easily react with the reducing substance during the hydrolysis, this result does not show that amino acids are not contained in the slime.

From the color reaction, it was assumed that uronic acid was contained. This was also ascertained by ion exchange resins. The results are shown in Table 4, and while the effluent of anion exchange resin did not reduce TTC

Table 4. Reducing activity of ion exchange resin effluent of slime hydrolysate.

	Original solution	Effluent of	
		Kation exchange resin	Anion exchange resin
Glucose value (mg/ml)	0.24	0.22	0.0

at all, that of the kation exchange resin was as active as the original solution. When dilute alkaline solution was passed through the anion exchange resin treated with the hydrolysate, the resulting solution become to reduce TTC. Accordingly, the reducing ability of the hydrolysate went along with anion, so the reducing component of the slime might be assumed to be uronic acid considering from those results.

c) Factors affecting viscosity of the slime solution. The relative viscosity of 0.1 per cent solution of the slime did not show any marked change with varying temperature. When temperature rises, the viscosity decreases markedly, but the relative viscosity, in turn, decreases only slightly.

One part of the slime solution was added to the seven parts of phosphate buffers of various reaction, and the resulting solutions were measured for their

Table 5. Temperature and viscosity of the slime solution.

Temperature (°C)		10	15	20	25	30	35	40
Flow time (sec)	Water	24.4	21.7	19.5	17.6	15.9	14.8	13.6
	0.1% solution	254.4	220.0	195.0	172.4	154.3	136.7	122.5
Relative viscosity		10.4	10.1	10.0	9.8	9.7	9.2	9.0

Table 6. Reaction and viscosity of the slime solution.

pH	7.9	7.1	6.1	5.2	3.9	3.2	2.1
Relative viscosity	2.49	2.41	2.44	2.48	2.40	2.28	1.85

viscosity. As shown in Table 6, the viscosity did not vary significantly between pH 4 and 8, while below 3 it slightly decreased. As shown in Table 2, the ash content of the slime is as high as 9.75 per cent and slime solution is heated in a strong acid solution only for 3 minutes, its viscosity declines to the same level of water. Then the presence of some metal is supposed to be a cause of the high viscosity, and detachment of the metallic kation may cause a decline of viscosity. Electrodialysis at 240 v is not successful to reduce ash content nor viscosity of the slime.

The viscosity of the slime solution at its various concentrations was listed in Table 7. Between the ranges of 12.5 mg and 0.5 mg of slime prepar-

Table 7. Concentration and viscosity of the slime solution.

Concentration (c) mg/100ml	100	50	12.5	6.3	3.2	1.6	0.8	0.4
Relative viscosity (η_r)	10.89	4.52	2.43	1.72	1.36	1.18	1.09	1.03
Specific viscosity (η_r-1)	9.89	3.52	1.43	0.72	0.36	0.18	0.09	0.03
$(\eta_r-1)/c\times10^4$	989	704	114	114	113	113	113	75

ration per 100 ml of the solution, $(\eta_r-1)/c$ was constant and thus the following formula was realized.

$$(\eta_r-1)/c=kM$$

M; Molecular weight, c; concentration, η_r; relative viscosity, k; constant.

Since the formula is applicable to the long chain molecule of the high molecular weight, the slime may be assumed to be a chain. Though inadequately, adopting the value of K about the cellulose in Schweizer's solution after Geoghegan (4), a gross molecular weight of the slime is computed as 22,500. Then, the slime is supposed to be a polymer of about 110 uronic

moieties. However, such a molecular weight is to be accurately estimated by an other appropriate method.

C) Preliminary Tests for the Growth experiments

As above mentioned, the organism shows a distinct induction period when shake-cultured. This would lead to a supposition that a nitrogen-free medium then used may not be adequate to its favorable growth, though the organism is a typical nitrogen fixer. Then, the growth in the N-free medium, composed of basal solution and glucose, was examined. The influence of glucose concentrations on the length of the induction period and growth rate was illustrated

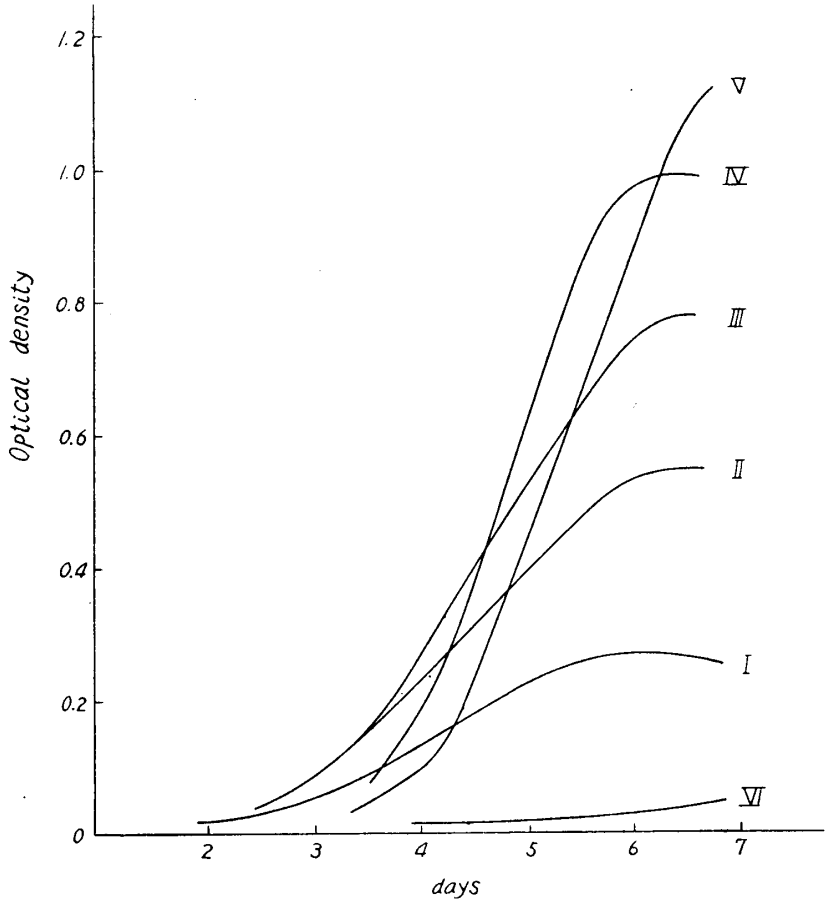

Fig. 4. Effect of glucose concentration on growth.
I: 0.5%, II: 1.0%, III: 1.5%,
IV: 2.0%, V: 5%, VI: 10%.

in Fig. 4. As the glucose concentration increased, the induction period became more prolonged while enhancing the growth rate. The glucose concentration over the range of 10 per cent inhibits the growth of the organism, and at 20 per cent no growth occurs. Therefore, 2 per cent glucose was adopted for the following works, since the induction period was distinct enough and the growth rate attained its maximum at this concentration.

The organism preferred rather a slightly acidic reaction as previously mentioned and grew even at pH 5.0, where, by contrast, other species of azotobacter failed to grow. After its full growth the reaction of the medium declined then to more acidic side. Opitimal initial pH was 6.4 and the induction period was shortened by one day without any variation in the growth rate.

Days of the induction period decreased proportionally to the logarithm of the drops of the inoculum, but the growth rate did not vary also in this case. The following formula may be realized:

$$x + a \log D = b$$

x; length of induction period expressed in days, D; drops of the inoculum, a and b; constants.

The experiments thereafter were performed using one drop a tube.

D) Growth in Glucose-bouillon and Koji-extract

The fact that the organism grew rapidly in the Koji-extract agar and did not grow on the glucose-bouillon agar at all was observed in the previous experiments. So the latter medium would be supposed to be deficient in a certain factor (s) such as growth factors and thus inadequate for the organism to utilize organic nitrogenous substances present in peptone or meat extract. Then this was examined.

In the Koji-extract the induction period was shortened by one day, and there occurred no growth in glucose-bouillon. This was also the case with glucose-bouillon supplemented with 1 per cent of purchased yeast extract as a source of growth factors. On the other hand, in the Koji extract, which had been preliminarily treated with 2 per cent of charcoal and thus supposed to be free from growth factors, the induction period was rather slightly shorter than in the untreated one. Dilution of the Koji-extract with the equal volume of water, also showed an enhancing effect, shortening the induction period further by one day. When the ordinary nitrogen fixers as *A. chroococcum*, *A. agile* and *A. vinelandii* were tested in like manner. They grew well in both glucose-bouillon and the charcoal-treated Koji-extract, though not in the untreated Koji-extract at all. This contradictory relationship is interesting, and will be examined in detail later. The growth factor may be inhibitory rather than enhancing the growth of the nitrogen fixers.

The glucose-bouillon was composed of glucose (1 per cent), peptone (1 per cent), meat extract (1 per cent), and NaCl (0.5 per cent). Insufficient supply of glucose was not the cause of the event, since the increase of glucose did not improve the situation. Various quantities of peptone and meat extract added to the glucose containing basal medium separately or simultaneously, and the growth in these media was examined. The results were illustrated in Fig. 5. Peptone shortens the induction period markedly, but its high con-

centration such as 4 per cent minimizes the growth. On the contrary, meat extract is rather inhibitory and 1 per cent of it permits the same level of growth as at 4 per cent of peptone. Peptone may reverse the inhibitory effect of meat extract(curve A2), since despite of the mixture of both components in the same level as the above glucose-bouillon A2 medium permits better growth.

So the salt, one of the components of the glucose-bouillon, was supposed also to be a cause. 50 mg per cent of salt inhibited the growth of the organism in the medium composed of 1 per cent of peptone, 1 per cent of meat extract, and 2 per cent of glucose, and 100 mg per cent of salt completely suppressed the growth. KCl exhibited the similar effects at the same level of Cl^- content as salt in the similar media. However, in the media such as containing amino acids mixtures, 100 mg per cent of Cl^- did not suppressed the growth of the organism, so Cl^- might help the inhibitory effect of meat extract.

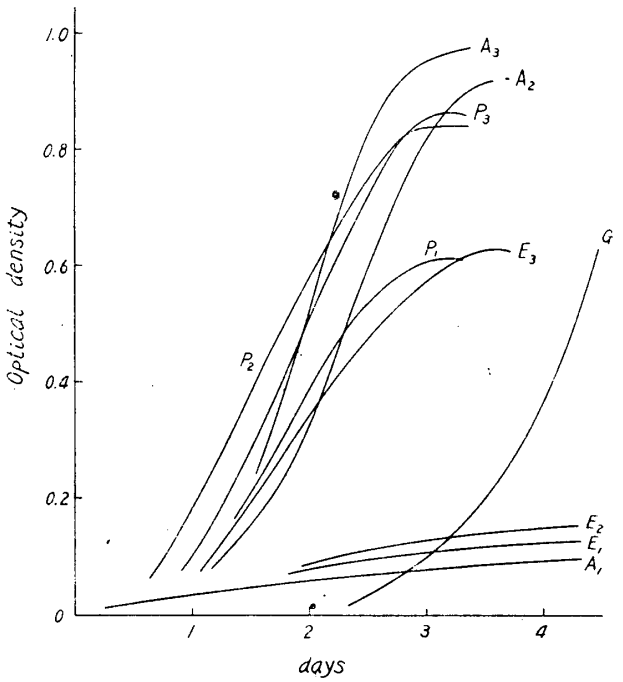

Fig. 5. Effect of peptone and meat extract
G : Basal medium
A_1 : G+Peptone (P) 2%+Meat Extreat (E) 2%
A_2 : G+P 1%+E 1%, A_3 : G+P 0.5%+E 0.5%
P_1 : G+P 4%, P_2 : G+P 2%, P_3 : G+P 1%
E_1 : G+E 4%, E_2 : G+E 2%, E_3 : G+E 1%

E) Growth in the Various Nitrogenous Media

It was very notable and interesting that the growth of the organism was largely promoted by the nitrogenous substance like peptone despite of its fixation capacity of atmospheric nitrogen. Then, other nitrogenous substances were tested. NH_4^+, NO_3^-, and the acid hydrolysate of vitamin-free casein, all were very stimulatory at the level of 50 mg per cent as nitrogen. Urea had no effect. Already 0.1 mg per cent of NH_4^+ and 1 mg per cent of casein hydrolysate as nitrogen markedly stimulated. On the other hand, 100 mg per cent of the former and 200 mg per cent of the latter suppressed the growth completely. Similarly amino acids mixture for lactobacilli suppressed the growth at the level of 50 mg per cent as nitrogen. So the so-called extra nitrogenous substances may have a significant effectiveness dependently on not only their qualitative character but also on their quantities added to the media.

Comparing the effectiveness of casein hydrolysate on the growth stimulation with that of NH_4^+-N, the latter is about ten fold more effective. On the contrary, the concentration of the former that suppressed the growth completely was 1.5 fold more. It may be easily supposed that amino acids cause their effects after decomposed to ammonia and other components by the organism. Minimal stimulatory concentration may be favorable to the supposition, however, minimal growth suppressing concentration is not. NH_4^+ is known to be a selective and powerful inhibitor of the nitrogen fixation, and amino acids may exhibit their effect on the process of amino acids synthesis and each acid may have its peculiar attacking site.

F) Effects of Amino Acids

From the point of view of the generally accepted inertness of azotobacters to the added amino acids, the above stimulatory or inhibitory effects of amino acids mixture were interesting. Molecular nitrogen which is at first fixed by the organism should be finally incorporated into the cellular proteins. During this process, the nitrogen may be transferred from the first fixation product to amino acids through some intermediates. It may be then supposed that the externally supplied amino acids might offer some disturbances to this incorporation process and thus resulting effectiveness visualized in the promotion and the inhibition of the growth. Then the effect of the individual amino acids was examined.

At first a series of ommision tests were performed. Amino acids mixture of the same composition as that employed in the bioassay with lactobacilli was prepared. Total nitrogen content was 50 mg per cent, among that 18 mg per cent was amino nitrogen and the remaining 32 mg per cent was ammonium nitrogen as NH_4Cl. When DL-alanine, L-histidine, L-tryptophan, DL-phenylalanine, L-cystine, or L-leucine was omitted respectively, there occurred no growth. On the other hand, when L-arginine, L-tyrosine, DL-methionine, or DL-serine was omitted respectively, the induction period was shortened markedly. Then the former six amino acids were combined and the omission tests thereof were again performed. In this case, amino nitrogen was 6 mg per cent and ammonium nitrogen was 32 mg per cent. In all cases, the growth was markedly promoted. This result stands against the previous one, and despite that these amino acids previously seemed to be an essential nutrilite, it denies such an essentiality. Then this fact would lead to another concept on the role of such external amino acids and it is persumably supposed that there are amino acids that are inhibitory to the organism when combined with ammonium nitrogen and the inhibitory effect is competitively reversed by other amino acids.

Then the 17 amino acids (each 18 mg per cent as nitrogen) were added singly or combined with 32 mg per cent of ammonium nitrogen added to the

basal medium, and the growth in the resulting media was examined. The results are summarized in Table 8. DL-threonine, DL-valine, DL-phenylalnine, DL-methionine, DL-serine, and glycine completely suppress the growth in both

Table 8. Effect of single amino acid on the growth of *A. indicum* sp.

Amino acid		Induction period	Growth rate
None	1	100	100
	2	65	500
Glutamic acid	1	100	250
	2	70	860
Aspartic acid	1	75	210
	2	70	470
Proline	1	100	250
	2	70	860
Tryptophan	1	125	300
	2	70	860
Leucine	1	65	260
	2	∞	—
Tyrosine	1	∞	—
	2	80	260
Lysine	1	80	380
	2	∞	—
Arginine	1	35	240
	2	65	170
Histidine	1	20	100
	2	50	100
Cystine	1	115	130

The numbers express the results in terms of the percentage of the nitrogen free basal medium. 1 contains no nitrogenous compound except corresponding amino acid. 2 Contains 18 mg per cent of NH_4Cl as nitrogen.

cases. While, L-glutamic acid, L-aspartic acid, L-proline, or L-tryptophan do not show any effect when combined with ammonium salt and shorten the induction period when added singly. Other amino acids are rather intermediary between above two groups, and DL-leucine as well as L-cystine abolish the growth when combined with ammonium salt and L-tyrosine does so when added singly.

From the omission tests it was made apparent that at the appropriate concentration, there are two groups of amino acids, that is, one is the inhibitors' group and the other is a group of competitive reversers of the former. On the other hand, from the results of addition tests amino acids are divided into a few groups. Between the groups of the two tests there is not any apparent close correlationship.

Discussion

A. indicum sp. investigated in the present work was isolated from the acidic volcanic ash soil in the northern district of Japan. To the contrary to the tropics only where this organism has been presumed to survive, this

Tohoku district is rather cool. So this species is assumed to be distributed widely beyond our expectation and if a suitable detecting technique is applied, such an assumption may be more conceivably verified. The organism utilizes butyrate well. When the soil plate supplemented with glucose is prepared and incubated, at first butyric acid is seemingly smelled and bubbles raise the surface of the soil plate and thereafter colonies of the azotobacter appear. Then it might be well mentioned that the organisms in the soil at first ferment the glucose and produce butyric acid which, in turn, azotobacter utilizes. This phenomenon may be a new evidence for a close interrelationship between clostridia and azotobacter. On the soil plate supplemented with butyrate, there appeared the colonies like those on the glucose supplemented plate. Other rapidly growing azotobacter such as *A. agile* may also utilize butyrate in preference to glucose, and thus butyrate may be a good substrate to isolate the nitrogen fixers. Furthermore, butyrate prevents the contamination of clostridia. On the other hand, benzoate has once been recommended by Winogradsky (5). On the benzoate agar, there appears a distinct pigment around colonies of some strains, and, however, *A. indicum* sp. is not able to utilize it. Therefore, benzoate may not be a comprehensive substrate for aerobic nitrogen fixers.

To be noteworthy, indicum strain cannot utilize acetate, succinate, and citrate. Acetate is readily oxidized by glucose grown *A. agile* and the latter two acids are after some induction period presumably caused by a permeability barrier. Such a distinct behavior of the indicum strain is of interest, however, there are many difficulties in researching the cell activities of this organism because of its abundunt slime production during the growth, which causes the separation of the cells from the culture to be difficult.

Most of the bacterial slimes are generally mixed polymers of sugars, amino sugars and uronic acids, except some simple polymers. There are a few polymers like dextran and levan that are composed of a single sugar, the former is composed of glucose while the latter of fructose. Stacey (6) has reported that the slime of *A. chroococcum* was mainly composed of glucose and uronic acid. There is no report of the bacterial slime that is mainly composed of an uronic acid like the present one. On the other hand, phyto-slimes such as pectinic acid and alginic acid are composed of galacturonic acid or manuronic acid respectively. So the concerned slime rather resembles that of the vegetative origin though its viscosity is much higher.

The slime was always produced by the organism in every nitrogen free medium even when supplemented with organic acids. Accordingly, the uronic moiety may not be derived directly from sugars supplied but rather synthesized from small molecules. In a medium containing some amino acid such as proline or so, on the other hand, the culture did not become viscous and cells

floculated. While, in either the casamino acid containing medium or Koji extract, the slime was always produced. Then it might be well assumed that some of the amino acids when singly added cause a prevention of the slime production, which, however, is reversed by a certain other amino acid. The slime is not the essential product of the cell metabolism.

All the results obtained on the influences of the extra amino acids upon the growth of the organism did not lead to any confirmation but rather confusion. This might be assumably contributory to the view that either of these amino acids so far tested more or less affects the intracellular nitrogen fixation process which is so feeble and sensitive to such externally supplied substances analogous to its essential intermediates that the cells would become more unstable to their environmental conditions supplied.

From the differences both in morphological and physiological characters between *A. indicum* and other species of *Azotobacteriaceae*, Derx (7) or Jensen (3) have presently proposed that the former is to be contained in a new genus *Beijerinckia.* Besides the diagnostic cited by them, other characters such as utilizability of mannose whilst non-utilizability of acetate, succinate as well as benzoate and also production of an unique slime composed of mainly an uronic acid might be comprised further. However, since an agreement with their proposal would require more detailed investigation of *Azotobacteriaceae*, at this time we should have somewhat reservation for a decided resolution of the problem. At any rate, *A. indicum* may play an important role in the Japanese soils because of its nitrogen fixation ability in acidic environment. Whether the interesting behaviors of this organism towards amino acids are also seen about other nitrogen fixers is to be the next program of our study. This subject is till now not so fully investigated as in other microorganisms depending solely on combined nitrogen.

Summary

1. Utilizability of carbon compounds by an acid tolerant nitrogen fixer, *Azotobacter indicum* sp., was examined. Glucose, mannose, galactose, fructose, sucrose, mannitol, lactate, butyrate, and pyruvate were utilizable. Pentoses, lactose, maltose, acetate, succinate and benzoate were not.

2. Slime of the organism, abundantly produced in nitrogen free media, was separated and shown to be composed of uronic acid solely. From the relation between intrinsic viscosity and concentration of its aqueous solution the shape of the slime was supposed to be a chain.

3. The organism grew gradually when stand cultivated, whilst on the contrary, it showed a marked induction period and grew rapidly when shake-cultivated. The induction period was shortened by the addition of a small amount of

nitrogenous compounds including amino acids which are generally accepted to be inert for the growth of other species of *Azotobacteriaceae*. And the large amount of these nitrogenous substances when supplied suppressed the growth completely.

4. Individual amino acids at an appropriate concentration had their own specific effects on the growth of the organism. There might be competition and cooperation among them.

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